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Short-term molecular-level effects of silver nanoparticle exposure on the earthworm, *Eisenia fetida*

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ABSTRACT

Short-term changes in levels of expression of nine stress response genes and oxidative damage of proteins were examined in *Eisenia fetida* exposed to polyvinylpyrrolidone (PVP) coated Ag nanoparticles (Ag-NP) and $AgNO_3$ in natural soils. The responses varied significantly among days with the highest number of significant changes occurring on day three. Similarity in gene expression patterns between Ag-NPs and $AgNO_3$ and significant relationships of expression of CAT and HSP70 with Ag soil concentration suggest similarity in toxicity mechanisms of Ag ions and NPs. Significant increases in the levels of protein carbonyls on day three of the exposure to both ions and Ag-NPs indicate that both treatments induced oxidative stress. Our results suggest that Ag ions drive short term toxicity of Ag-NPs in *E. fetida*. However, given that <15% of Ag in the NPs was oxidized in these soils, dissolution of Ag-NPs is likely to occur after or during their uptake.

1. Introduction

In recent years there has been a dramatic increase in production of products containing Ag-nanoparticles (Ag-NPs) and the release of Ag-NPs into the environment is likely already occurring (Blaser et al., 2008; Luoma, 2008). One of their major routes of entry into the environment is predicted to be through wastewater treatments plants, where 90% of Ag-NPs are likely to be partitioned to sewage sludge (Gottschalk et al., 2009; Mueller and Nowack, 2008) the majority of which is applied to agricultural lands as biosolids in many locations (USEPA, 2009). These sludge amended soils containing Ag-NPs present potential risk to the health of terrestrial organisms and may gain entry into food webs (Judy et al., 2010; Shoults-Wilson et al., 2011a, 2011b, 2011c).

Our recent studies on the effects of Ag-NPs and Ag ions on earthworm, *Eisenia fetida*, demonstrated a decrease in reproduction after exposure to $AgNO_3$ in soil at concentrations of about 100 mg/kg for 28 days, which is similar to the highest Ag concentrations currently expected in sewage sludge (USEPA, 2009), while adverse effects on reproduction from Ag-NPs were documented at about 800 mg/kg (Shoults-Wilson et al., 2011a, 2011b). In our previous studies, we concluded that reproductive toxicity was likely due to release of free Ag ions from Ag-NPs based on extended X-ray absorption fine structure spectroscopy (EXAFS) of soil samples showing that the Ag (I) fraction was similar to the total Ag concentration at which reproductive effects were observed in the $AgNO_3$ treatment. However, when we examined a more sensitive endpoint, avoidance behavior, earthworms showed a significant response after 48 h of exposure to Ag-NPs (Shoults-Wilson et al., 2011c) at Ag concentrations of about 10 mg/kg, which are relevant to the concentrations predicted in sludge (Gottschalk et al., 2009). The earthworms responded at similar concentrations for Ag-NPs and ions at 48 h; however, there was an initial (immediate) avoidance response for ions but not Ag-NPs, suggesting a delay in onset of action for Ag-NPs that did not occur for $AgNO_3$. Similar sensitivity at 48 h was observed despite the fact that at least 90% of the Ag in the Ag-NP treatment remained in zero-valent form, indicating mostly intact Ag-NPs (Shoults-Wilson et al., 2011a, 2011b). Changes in the microbial community that may have led to such avoidance behavior were also ruled out (Shoults-Wilson et al., 2011c). Based on these lines of evidence, we hypothesized that the avoidance response was a particle specific, and not related to dissolution of the Ag-NPs in the soil matrix.

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In order to test the hypothesis that Ag-NPs act through toxicity mechanisms that are distinct from those of $AgNO_3$, we investigated short-term changes in gene expression for genes that have been demonstrated to respond to toxic metal ions and/or oxidative stress (Brulle et al., 2006, 2007; Burgos et al., 2005; Homa et al., 2005; Kiliç, 2011; Spurgeon et al., 2004; Unrine et al., 2010a; Zhang et al., 2009) as well as changes in oxidative damage of proteins (Drake et al., 2003; Hardas et al., 2010; Yokel et al., 2009). We expected that if the particles and ions acted through similar mechanisms, the patterns of changes in gene expression and oxidative damage would also be similar. Our previous studies have also indicated that intact metallic NPs can induce specific changes in patterns of gene expression in another terrestrial invertebrate, *Caenorhabditis elegans*, which indicate possible mechanisms of toxicity (Tsyusko et al., 2012). Oxidative stress is a putative mechanism whereby intact Ag-NPs may cause toxicity in terrestrial invertebrates (Lim et al., 2012; Yang et al., 2011). Because *E. fetida* has not had its genome completely sequenced and published at the time the study was conducted, we were limited to examination of a few genes. The genes selected from the available sequences at GenBank were associated with metal homeostasis – metallothionein (MT) (Stürzenbaum et al., 1998), metal ion binding and protein regulating – calmodulin (CaM) (Ouyang and Vogel, 1998), general stress and macromolecular damage – ubiquitin and heat shock proteins 60, 70 (HSP60, HSP70) (Flick and Kaiser, 2012; Parsell and Lindquist, 1993), and oxidative stress – catalase (CAT), protein kinase C2 (PKC), superoxide dismutase (SOD), and glutathione-S-transferase (GST) (Di Giulio et al., 1995; Gopalakrishna and Jaken, 2000). We also examined oxidative damage to proteins by measuring protein carbonyls (PC), protein-bound 4-hydroxy 2-transnonenal (HNE), and protein-resident 3-nitrotyrosine (3-NT). These biomarkers have shown responses to oxidative stress caused by CeO_2 NPs (Hardas et al., 2010; Yokel et al., 2009).

Eisenia fetida were exposed in a natural soil to Ag-NPs and $AgNO_3$ for three different short time periods. The samples were collected from a previous study that investigated the influence of particle size and soil type on Ag uptake from Ag-NPs (Shoults-Wilson et al., 2011a). The effect of particle size was also investigated by comparing responses resulting from exposures to Ag-NPs with two different size distributions. In addition, the responses to Ag-NPs of the same size but with two different concentrations were compared to determine whether gene expression levels vary significantly by concentration.

2. Materials and methods

2.1. Material characterization

Two Ag-NPs with polyvinylpyrrolidone (PVP) coating with nominal sizes of 10 (SNP) and 30–50 (LNP) nm were purchased from NanoAmor (Nanostructured & Amorphous Materials, Houston, TX). Primary particle size was determined via transmission electron microscopy (TEM). The particle hydrodynamic radius in aqueous suspension and zeta potential were measured using dynamic light scattering (DLS) and phase analysis light scattering (PALS) with a Malvern Zeta-Sizer Nano-ZS (Malvern Instruments, Malvern, UK). Complete details of the particle characterization can be found in Shoults-Wilson et al. (2011a). Briefly, the SNP had a primary particle (TEM) diameter of 40.9 ± 0.8 nm (mean ± standard error of the mean) and the LNP had a mean primary particle diameter of 56.4 ± 1.2 nm. However, dynamic light scattering in 18 MΩ deionized (DI) water after sonication indicated a volume weighted geometric mean diameter of 10 nm for the SNP versus 30 nm for the LNP, indicating possible effects of drying during the TEM analysis on the particle size distribution. The apparent ζ potential in DI water (Hückel approximation) was −49.5 ± 1.7 mV for the SNP and −35.9 ± 0.8 mV for the LNP. Both the SNP and the LNP were approximately 90% Ag (0) and 10% Ag_2O.

2.2. Measuring Ag soil concentrations

Soil samples from exposure chambers were dried at 60 °C and microwave digested in sealed Teflon bombs at 180 °C in concentrated trace-metal grade HNO_3 (CEM MarsXpress, Matthews, NC, USA). Ag concentrations in diluted digestates were analyzed by ICP-MS (Agilent 7500 cx, Santa Clara, CA USA). Each digestion set contained reagent blanks and standard reference soils (National Institute of Standards and Technology, Standard Reference Material 2709). Details of the validation of this method specifically for Ag-NPs, have been previously described (Shoults-Wilson et al., 2011a).

2.3. Exposures

The exposure protocol has been described previously where we report data on uptake of Ag from the same exposures (Shoults-Wilson et al., 2011a). Briefly, all exposures to Ag-NPs and $AgNO_3$ were performed in Yeager sandy loam (YSL), a soil from a site in central Kentucky (Estill County) with 1.77% of organic matter and 9.18 cmol/kg for cation exchange capacity, which was air dried and sieved. More details on soil properties, are reported in (Table 1) and in Shoults-Wilson et al. (2011a). Ag-NPs were sonicated for 15 min in 18 MΩ DI water and the suspensions were applied to soils at a rate of 50% water holding capacity. The nominal concentration of Ag used for NPs of both sizes (SNP and LNP) and for $AgNO_3$ was 100 mg/kg dry soil. To investigate changes in gene expression in the worms exposed to low versus high Ag soil concentration we have also included a treatment with nominal concentration of 500 mg/kg dry soil for LNP (high LNP). These soil concentrations were relevant to both short-term effects on avoidance behavior as well as long-term effects on reproduction, growth and survival (Shoults-Wilson et al., 2011a, 2011c). Twelve adult worms per treatment were selected in a size-stratified manner (to include a representative sample of worm sizes). There were a total of 4 treatments including control, small Ag-NP (SNP), large Ag-NPs (LNP) and $AgNO_3$. The treatment for LNP included two nominal concentrations of 100 (LNP low) and 500 (LNP high) mg/kg with 12 worms for each concentration. The worms were maintained at 20 °C with 12 light hours per day. Four individual worms per treatment (one from each container) were randomly selected for qRT-PCR and protein analysis after 0, 1, 3, and 7 days of exposure, flash frozen in liquid nitrogen and stored at −80 °C until analyzed.

2.4. RNA extractions and qRT-PCR

RNA was extracted from all worms after crushing them in mortar with pestle in liquid nitrogen and using Trizol (Invitrogen) followed by purification with Qiagen RNeasy Kit (Qiagen, Chatsworth, CA). All RNA samples were analyzed using microfluidic electrophoresis (Agilent 2100 Bioanalyzer), as well as reading absorbance at 260 and 280 nm using a spectrophotometer equipped with a nL volume cuvette. All samples showed no signs of degradation with RINs (RNA Integrity Number) of 9 and 10. High capacity RNA-to-cDNA Master Mix (Applied Biosystems) was used to convert total RNA of 500 ng into cDNA for all samples. Nine stress response genes including CaM 16-1 (DQ286708.1), CAT (DQ286713), PKC2 (DQ286717), MT (AJ236886), SOD (DQ286712), HSP60 (DQ286710), HSP70 (DQ286711), UBQ- (DQ286715), and pi-class GST (GQ865565) were used for qRT-PCR. All primers and probes were designed using Primer Express (Applied Biosystems) with the sequences already deposited in the GenBank except for GST and β-actin (reference gene). We were unable to amplify the product using initial qRT-PCR primers and probes designed from the available GenBank sequences for GST (GQ865565) and β-actin (DQ286722). Instead we re-designed primers for sequencing and sequenced the regions of the interest using our samples from different treatments (SNP, LNP, $AgNO_3$, and control). The sequences were deposited into the GenBank (Accession # for β-actin – JQ038870 and for GST – JQ038871). After blasting the new sequences against the GenBank sequences and using only conserved regions, we successfully re-designed primers and probes for these two genes. All primers and TaqMan probes were purchased from Applied Biosystems. The β-actin gene did not show any significant changes among the treatments and was selected as a reference gene. The qRT-PCR was performed in 10 μl reaction using 1× FastStart TaqMan Master Mix, 300 nM of TaqMan primers and probe, and 2 μl of diluted 1:9 cDNA. The list of primers and probes with their corresponding efficiencies (96–101%) and the gel image for their amplification product are presented in Table 2 and Fig. S2. Amplification for all genes was performed using StepOne Plus System (Applied Biosystems)

Table 1
Nominal and measured Ag concentrations and pH of the soils used for exposures of the earthworms to ionic and nanoparticulate Ag to examine their short-term molecular-level responses. BDL, Below detection limit.

ID	Ag type	Particle size (nm)	Particle coating	Nominal $[Ag]_{soil}$ (mg/kg)	$[Ag]_{soil}$ (mg/kg)	pH
Control	NA	NA	NA	0	BDL	6.7
$AgNO_3$	Ion	NA	NA	100	87.9	6.8
SNP low	NP	10	PVP	100	119.0	6.9
LNP low	NP	30–50	PVP	100	61.6	6.8
LNP high	NP	30–50	PVP	500	288.0	6.7

Table 2
qRT-PCR primers and probes for TaqMan assays used to examine short-term molecular-level responses of earthworms, *Eisenia fetida*, exposed to Ag ions and Ag nanoparticles. The accession number is the number corresponding to the sequences used to design these assays. Assay ID is the ID that can be used to order assay from Applied Biosystems.

Gene name	Assay ID (Applied Biosystems)	Forward primer sequence	Reverse primer sequence	Probe sequence
β-Actin	AICSUU9	TCCACCTTCCAGCAGATGTG	GCACTTTCTGTGGACGATGGAT	CAGCAAGCAGGAGTAC
CaM	AIX0ZPZ	GGCTGCTGAGCTGAGACATG	CACCTCTTCGTCCGTAAGCTTCT	CATGACCAACCTTGGC
CAT	AIPACK7	ACGCCGACGGAGAAGCT	TGCCTTGGTTGGTCTTGTGA	TGTACTGCAAGTTCC
PKC	AIQJARF	TGCGCTGTCCGCAAAA	TCTGTTTGGAAGCAGGAGTGAA	CCCGTACCTAACTGC
MT	AIWR1JR	GCTGTGCTGACGCTGAGAAG	CCAGCCGAGCATTTGCA	CAAATGTGGAAATGCA
HSP60	AIWR1OY	AGGCTGGTGCAGGATGTTG	CGTTGTTCCGTCTCCTGCTT	CAACAGCACAAATGA
HSP70	AIX0ZU6	GATCGAGGTGACATTCGACATC	CGACAGCCGACACGTTCA	CGCCAACGGCATC
UBQ	AI1RUDU	CGTCTTCGTGGAGGTATGCA	TCAACTTCTAGGGTGATGGTCTTTC	CTTCGTGAAGACCTTG
SOD	AIRR8XN	GCGATAACACAAATGGTTGCA	TGAGTCTTTCCAAATGGGTTGA	AAGTGCAGGTGCTCAC
GST	AID1S7W	GGAGGCGCCGGATTCT	AGATCAAAGATGGTGTAGTCAACAAAA	TTGGCGACAAGATC

for 10 min at 95 °C, followed by 40 temperature cycles for 15 s at 95 °C and for 1 min at 60 °C. All samples for every gene were run in triplicates. Negatives and reverse transcription (RT) negatives were run for every gene/sample to check for DNA contamination. The data were exported into REST software, and gene expression levels were calculated relative to reference gene, *β-actin*, and relative to controls according to Pfaffl et al. (2002).

2.5. Analysis of oxidative damage to proteins

Aliquots of frozen and ground earthworm tissues were also analyzed for protein oxidative damage. Three endpoints were studied: (1) Protein carbonyls (PC; a product of reactive oxygen species oxidation of protein amino acid side chains, e.g., Lysine, Arginine, Proline, Threonine, and Histidine; peptide backbone scission; Michael addition reactions of His, Lys and Cys residues with products of lipid peroxidation; or glycoxidation reactions); (2) 3-nitrotyrosine (protein-resident 3-NT; a covalent protein modification from the action of reactive nitrogen species on the ortho position of the aromatic ring of tyrosine residues in proteins); and (3) protein-bound (HNE; a product of lipid peroxidation of polyunsaturated omega-6 acyl groups, such as arachidonic, or linoleic groups on glycerophospholipids and corresponding fatty acids). Each parameter was determined by the slot-blot technique with specific antibodies towards the oxidative modification. Each sample was analyzed in multiple replicates, and compared to samples from control earthworms processed in the same analysis. Details of the methods have been previously described (Butterfield, 1997; Sultana et al., 2005).

2.6. Enzyme activity assay for catalase

Enzyme activity assay for catalase activity was performed using a commercially available kit obtained from Cayman Chemical Company (Ann Arbor, MI). In brief, 1 μg of each sample was loaded on 96-well plate and subsequently mixed with assay buffers and reagents provided with the kit, as per the given instructions. The enzymatic reaction was monitored spectrophotometrically and the absorbance was measured at wavelength 540 nm.

2.7. Statistical methods

Significance of up- or down-regulation of genes relative to controls was tested in REST (Pfaffl et al., 2002). We used SAS v9.2 (SAS Institute, Cary, NC, USA) for all other statistical tests. The gene expression data were normalized to controls and were logarithmically transformed to satisfy the normality assumption for parametric statistics using GenEx data analysis software (MultiD, Sweden). All data sets were tested for outliers using Dixon's Q-test (Alfassi et al., 2005). When calculated Q-values of the suspected outliers exceeded Q-critical values (90% confidence interval), these extreme values were removed. Least squares linear regression analyses were conducted for each gene to examine relationships between relative gene expression and measured Ag concentration in soils for each of the exposure days (day 1, 3, and 7). All comparisons were conducted among treatments with the same nominal Ag soil concentration of 100 mg/kg and also between LNP of the same size (30–50 nm) but with two different soil concentrations, 100 mg/kg (low LNP) and 500 mg/kg (high LNP). MANOVA was used to investigate the overall effects of day tested, treatment type ($AgNO_3$, SNP, LNP low, LNP high), and the interaction of the day tested and the treatment type on gene expression normalized to controls (Table S1). Two-way ANOVAs were then used to investigate the main effects of day tested, and treatment type ($AgNO_3$, SNP, LNP low, LNP high) on gene expression normalized to controls (Table S2). If the MANOVA results were not significant for the interaction term, the interaction term of the day and the treatment type was not included in the model for the two-way ANOVAs for the gene expression data. ANOVAs with and without interaction were performed for PC, HNE, and 3-NT data. In order to make further comparisons between means, Tukey's multiple comparisons were performed ($\alpha = 0.05$). Principal Component Analysis (PCA) was also conducted for each day. Biplots to examine relationships between the 1st and 2nd principal components were examined to visualize multivariate treatment differences (Fig. S3).

3. Results and discussion

The actual concentrations of Ag measured in soils and pH of the treatments are presented in Table 1. The measured Ag soil concentrations differed from the nominal concentrations due to inhomogeneity of dosing suspensions which were prepared by dispersing powders. However, having this range of Ag concentrations presented additional opportunities to examine effects across concentration gradient which varied from 62 to 288 mg/kg (Table 1).

We observed dramatic changes in gene expression for several genes in all treatments as a function of exposure time. Changes in gene expression levels and in levels of PC in response to $AgNO_3$ and Ag-NPs varied significantly among days (Figs. 1 and 2). Seven (CaM 16-1, CAT, MT, SOD, HSP60, HSP70, and GST) out of nine analyzed genes had day as a statistically significant factor determining changes in gene expression (Table S2). The third day of the exposure showed the highest number ($n = 15$) of significant changes in gene expression within treatments relative to controls followed by 7th day, where 10 significant changes in expression levels were observed (as shown in Fig. 1). Significant temporal variation in gene expression in response to Ag-NPs exposure has also been observed in Japanese medaka (Chae et al., 2009) where, for instance, increase in levels of MT expression were demonstrated up to two days after exposure but the expression levels decreased to control levels after four days. Taken together with our results, it appears that MT may be primarily involved in short term detoxification mechanisms for Ag, although for the LNP high exposure concentration, MT remained significantly up-regulated on day 7. The results highlight the transience of molecular level responses during initial exposure to Ag and Ag-NPs.

There were similar patterns of changes in expression of several genes among earthworms treated with SNP, LNP and $AgNO_3$, suggesting similarity in the mechanism of toxicity when exposure time and concentration are similar. For example, significant up-regulation of MT, on day three, HSP60 on day seven and significant down-regulation of CaM three days after the exposure were documented across all treatments (Fig. 1). There were only a few instances where a gene did not show a significant response to Ag ions but its expression was significantly changed in at least one Ag-NP treatment. Protein kinase C was only significantly down-regulated in the LNP low and LNP high treatments on day seven. Although not statistically significant, a similar magnitude of down-regulation was observed in SNP treatment. There were also no significant differences among treatments with similar nominal Ag soil concentrations. In addition, the first two principal components did not differentiate among treatments at any time of the exposure

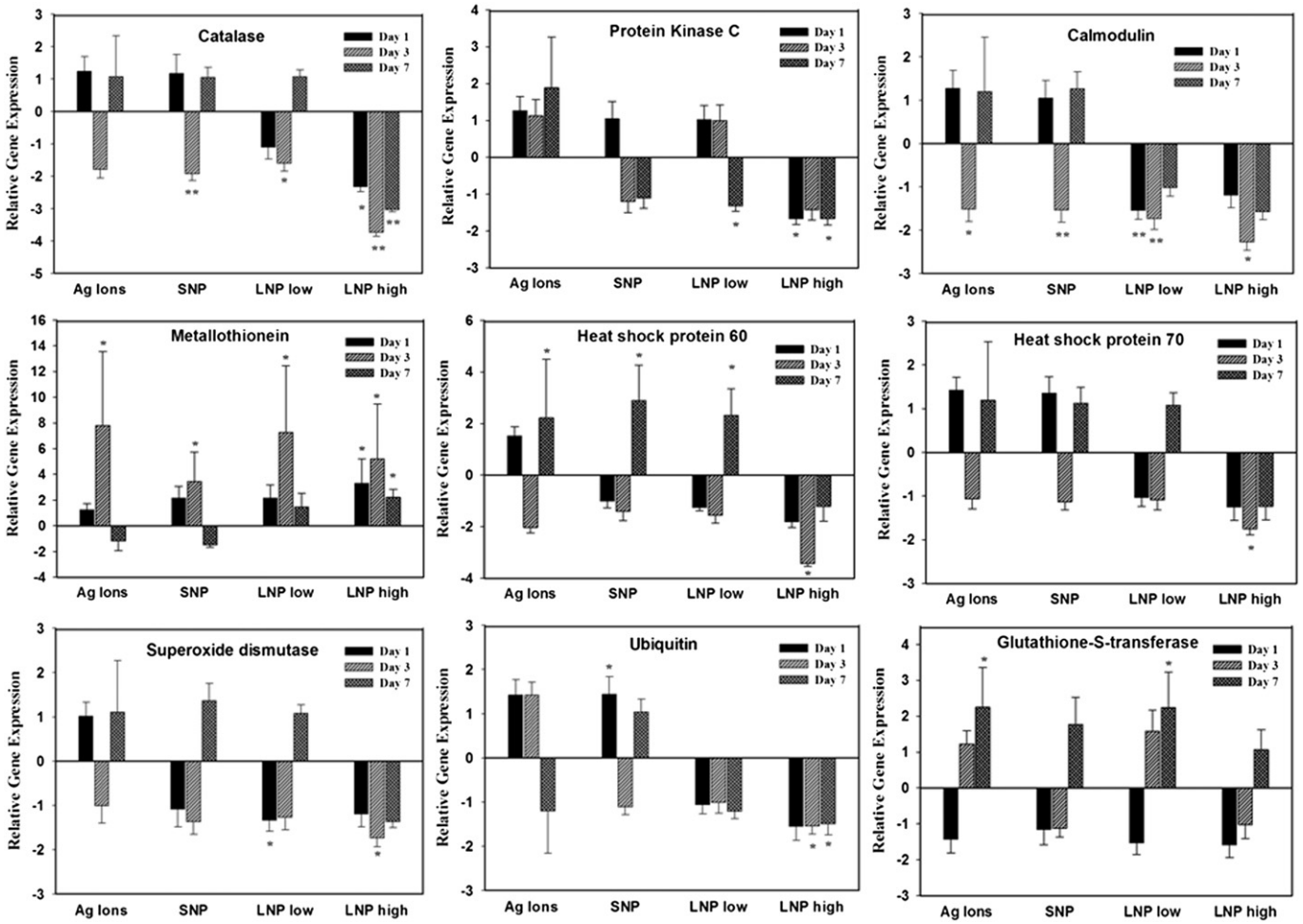

Fig. 1. Relative gene expression levels (normalized to β-actin) of nine stress response genes in *Eisenia fetida* exposed to $AgNO_3$ and Ag nanoparticles (Ag-NPs; small-SNP and large-LNP) for one, three, and seven days. LNP were used at low (LNP low) and high (LNP high) Ag concentration of 100 and 500 mg/kg. The expression levels significantly different from controls within treatments are shown by $^{*}0.001 < p < 0.05$ and by $^{**}p < 0.001$.

(Fig. S3). Catalase was also significantly down-regulated in response to both SNP and LNPs but not to Ag ions on day three. Even though the change in CAT expression on day three for Ag ions was not significantly different from controls, the magnitude of its down-regulation was similar to that of SNP and LNP low. When a higher concentration was tested (LNP high), significant down-regulation of CAT was observed on all three days (Fig. 1) and it was also significantly lower than the LNP low treatment (Table S2). Catalase enzyme activity was also significantly decreased on day three for Ag ions and SNP (Fig. 3, Table S4) and even though not

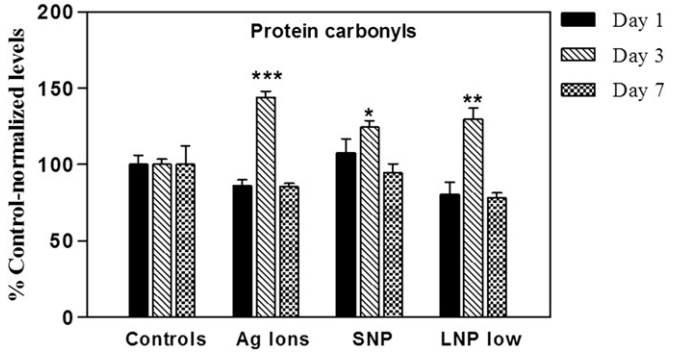

Fig. 2. Levels of protein carbonyls measured in *E. fetida* after exposure to $AgNO_3$ and Ag nanoparticles (Ag-NPs; small-SNP and large-LNP) for one, three, and seven days are expressed as % control (mean ± SEM). The treatments that are significantly different from controls are shown by $^{***}p < 0.001$, $^{**}0.001 < p < 0.01$, and $^{*}0.01 < p < 0.05$.

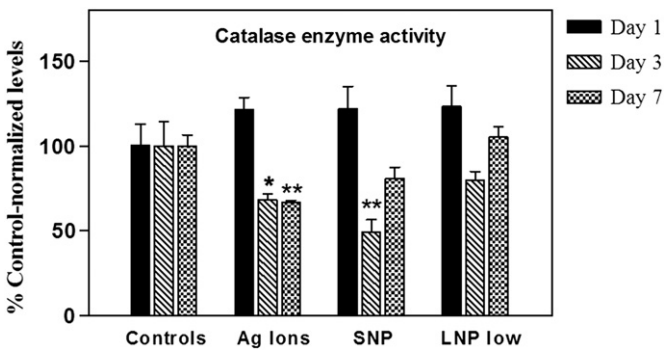

Fig. 3. Catalase enzyme activity measured in *Esenia fetida* after exposure to $AgNO_3$ and Ag nanoparticles (Ag-NPs; small-SNP and large-LNP) for one, three, and seven days are expressed as % control (mean ± SEM). The treatments that are significantly different from controls are shown by $^{***}p < 0.001$, $^{**}0.001 < p < 0.01$, and $^{*}0.01 < p < 0.05$.

significant, a similar magnitude of down-regulation was observed for LNP on day 3 (Fig. 3). In some instances, NP size seemed to also be an important factor. For example, CaM was up-regulated on day one, followed by significant down regulation on day three, and up-regulation on day seven for Ag ions and SNP. However, for LNP low, CaM was significantly down-regulated on both days one and three (Fig. 1). Taken together, the data do not provide the overall impression that there were differences in gene expression between $AgNO_3$ and Ag-NPs, since no unique responses could be found that were specific to either particles or ions.

The responses of two genes could be shown to be directly related to total Ag soil concentration regardless of whether Ag was present as ions or in nanoparticulate form. Significant correlations of gene expression with total Ag soil concentrations were observed for two genes (CAT and HSP70 in Fig. 4). Catalase and HSP70 expression levels significantly decreased with increased Ag soil concentration ($r^2 = 0.99$ $p = 0.005$ for CAT and $r^2 = 0.95$ $p = 0.02$ for HSP70, Fig. 4) on day three. Significant correlations calculated using the data from all treatments from day three suggest that gene expression levels for these two genes are determined by Ag soil concentration independent of the NP size or Ag form (NPs or ions). On the other hand, our previous study (Shoults-Wilson et al., 2011a) demonstrated that after up to 28 days, about 85% of the Ag in the NP treated soil remained in the zero-valent state, indicating intact NPs. The study also showed that uptake of Ag at similar nominal exposure concentrations was similar among $AgNO_3$, SNP and LNP over the first seven days of exposure (Shoults-Wilson et al., 2011a). The only explanation that reconciles these strong correlations after three days with the known speciation of Ag in the soil and similarity in uptake is that the Ag-NPs are taken up and dissolved, releasing Ag ions within the tissues. Our previous studies have demonstrated that either intact or oxidized metallic NPs can be taken up and distributed within earthworm tissues (Unrine et al., 2010a, 2010b). This could also explain the 48 h delay observed in the avoidance response to Ag-NPs relative to Ag ions (Shoults-Wilson et al., 2011c).

Among the markers of oxidative damage to proteins, protein carbonyls showed a significant increase in their levels for all treatments relative to controls on day three, while 3NT and HNE levels were significantly decreased on day seven in response to $AgNO_3$ and LNP for 3NT and $AgNO_3$ for HNE (Figs. 2 and S1, Table S3). These significant decreases in 3NT and HNE levels suggest possible activation of detoxification and repair mechanisms by day seven in response to the exposure of ions and LNPs. It is important to note that the measured Ag soil concentration (Table 1) was lower for LNP than for SNP and this may explain the difference in responses in 3NT and HNE levels between these two types of NPs. The significant increases in the levels of protein carbonyls caused by ions and both NPs (Fig. 2) on day three indicate that the earthworms undergo oxidative stress as a result of both Ag-NP and Ag ion exposure. It is often repeated that oxidative stress is a nanoparticle specific mode of action (Nel et al., 2009; Yang et al., 2011); however, it is a mode of action for many toxicants including metal ions (Valko et al., 2005). A significant decrease in gene expression levels of CAT and in its enzyme activity also occurs three days after the exposure to Ag-NPs (Figs. 1 and 3). Catalase is responsible for metabolizing the highly reactive oxygen species, hydrogen peroxide. Because of its down-regulation and decreased enzyme activity, CAT may not be efficient in degradation of H_2O_2 leading to accumulation of H_2O_2 which may be responsible for the increased levels of protein carbonyls. We only observed increases in PC on day three, where significant down-regulation of CAT was also documented (Fig. 1). Although it is not clear why CAT would be down-regulated in response to Ag exposure, other studies have documented CAT down-regulation in response to metal ion exposure (Bigot et al., 2011) and Ag-NPs (Chae et al., 2009; Choi et al., 2010).

Currently there are a number of in-vitro and in-vivo studies examining mechanisms and comparing genotoxic effects of Ag-NPs and Ag ions. For instance, in vitro study with human hepatoma cells demonstrated induced expression of SOD1, glutathione peroxidase (GPx1), and CAT by both Ag ions and Ag-NPs while only ions induced MT 1B (Kim et al., 2009). In another in vitro study using human intestinal epithelium cells, Ag-NPs and ions showed similar responses in global gene expressions associated with oxidative and endoplasmic reticulum stress (Bouwmeester et al., 2011). Most of the in-vivo studies with Ag-NPs and Ag ions were conducted in aquatic exposures with medaka (*Oryzias latipes* (Chae et al., 2009)), zebrafish (Choi et al., 2010), and nematode (*Caenorhabditis elegans* (Roh et al., 2009)) with exception of *Drosophila melanogaster* (Ahamed et al., 2010) and studies in mice (Park et al., 2011). Chae et al. (2009) observed significant differences between ions and Ag-NPs in expression patterns of five fish biomarkers, with responses from HSP70, p53, and transferrin (TF) being the most pronounced. In zebrafish, where Choi et al. (2010) performed exposures in solutions presumably free of Ag ions (deionized with ion exchange resin), Ag-NPs induced expression of MT-2, the levels of malondialdehyde (byproduct of cellular lipid peroxidation), and total glutathione, while expression levels of CAT and GPx were reduced. Although the ion exchange resin may have removed Ag ions from the exposure media, dissolution of Ag-NPs in tissues cannot be ruled out. In *C. elegans* (Roh et al., 2009) the exposure to

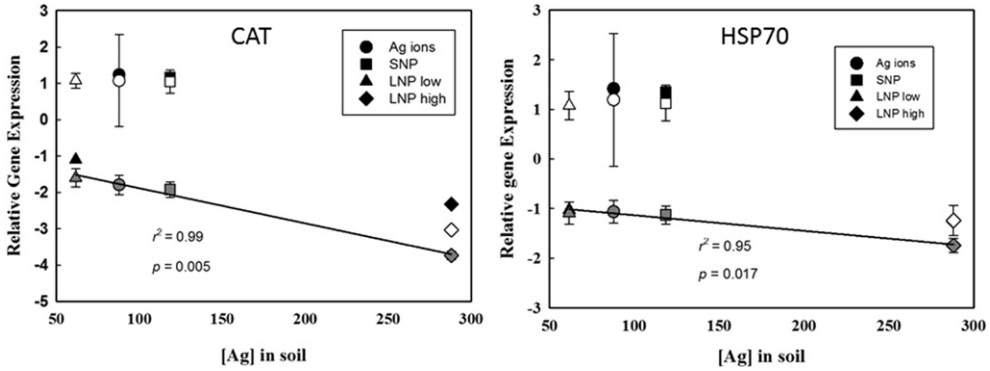

Fig. 4. Relationships between gene expression levels for catalase (CAT) and heat shock protein 70 (HSP70) and measured Ag soil concentration for *Eisenia fetida* exposed to $AgNO_3$ and Ag nanoparticles (Ag-NPs; small-SNP and large-LNP) for one (black), three (gray), and seven (white) days. LNP are shown for nominal Ag concentration of 100 (low LNP) and 500 (high LNP) mg/kg.

Ag ions at 0.1 and 0.5 mg/L resulted in two-fold induction of HSP gene groups, while exposure to Ag-NPs induced MT-2, SOD-3, and DAF-12. Another *C. elegans* study with oxidative stress- and metal-sensitive mutant strains (Yang et al., 2011) demonstrated increased sensitivity to both ions and NPs for the metal-sensitive mutants (MT-2 and PCS-1) but oxidative stress-sensitive mutants (MEV-1 and SOD-3) showed sensitivity only to Ag-NPs. Similarly to aquatic studies, *Drosophila melanogaster* fed with cornmeal dosed with Ag-NPs (Ahamed et al., 2010) showed signs of oxidative stress, heat shock stress, DNA damage and apoptosis. In contrast to the aquatic and our studies, the levels of CAT were significantly induced by Ag-NPs in *D. melanogaster* suggesting organism specific responses to Ag-NPs. These results from in vivo and in vitro studies demonstrate that organisms experience various type of stress during the exposures to both Ag ions and Ag-NPs. Comparisons of genetic signatures resulting from the exposures to Ag ions and Ag-NPs in these studies revealed similarities, indicating that the effects can be explained by dissolution of Ag-NPs, and also differences, which are suggestive of particle-specific effects.

The results from this earthworm study taken together with our previous results on Ag speciation in the soils (Shoults-Wilson et al., 2011a), suggest that the differences in mechanisms of short term (<7 days) toxicity of Ag-NPs versus Ag ions may only be related to the mechanism of delivery of Ag to cells. The rate of Ag uptake and internalization may differ significantly between Ag ions and Ag-NPs; however, it appears that their toxicodynamics once within the cells may be similar, with intracellular Ag ions driving the effects. These results help to explain why we observed avoidance of Ag-NPs and $AgNO_3$ in our previous studies at similar concentrations, but with differences in the timing of effects. On the other hand, they do not explain why much higher concentrations of Ag-NPs are required to elicit reproductive effects in *E. fetida* for 28 day exposures. Our previous study (Shoults-Wilson et al., 2011a) showed that uptake of Ag from $AgNO_3$ treated soil is greater than from Ag-NP treated soil over long-term exposure. We have observed similar disparities in uptake when comparing $HAuCl_4$ to Au-NPs and $CuSO_4$ to Cu-NPs (Unrine et al., 2010a, 2010b). It is possible that these differences in uptake are more significant in terms of sensitivity for sub-chronic endpoints, such as reproduction, than for the acute endpoints examined here and in our behavioral avoidance experiments. The transient down-regulation of CAT and subsequent decrease in its enzyme activity may also play a role. If a decrease in CAT enzyme activity leads to accumulation of H_2O_2, such accumulation may help to accelerate the dissolution of Ag-NPs in cells (Ho et al., 2010), particularly within lysosomes or other intracellular vesicles. If down-regulation and decreased CAT enzyme activity do not persist, as our data suggest, then it is possible that dissolution of the Ag-NPs is more rapid during the onset of exposure than over longer time period. This may help to explain why the difference in sensitivity between Ag-NPs and $AgNO_3$ is so much greater for longer time points than for short-term exposure since the in tissue particle dissolution rate may decrease over time. The results of this study highlight the need for caution when predicting long-term ecological consequences of Ag-NP exposure from short-term biochemical or behavioral responses. Future studies should investigate the in-tissue dissolution of Ag-NPs. Unfortunately, this is an extremely challenging task. Our previous studies used synchrotron-based X-ray microspectroscopy to investigate in-situ speciation of Cu and Au-NPs within tissues (Unrine et al., 2010a, 2010b). However, due to the low concentrations relevant to Ag toxicity (low μg/g), such approaches remain a major challenge for Ag-NPs, since few instruments exist with micron resolution and detection limits that enable spectroscopy at low ug/g concentrations.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envpol.2012.08.003.

References

Ahamed, M., Posgai, R., Gorey, T.J., Nielsen, M., Hussain, S.M., Rowe, J.J., 2010. Silver nanoparticles induced heat shock protein 70, oxidative stress and apoptosis in *Drosophila melanogaster*. Toxicology and Applied Pharmacology 242, 263–269.

Alfassi, Z.B., Boger, Z., Ronen, Y., 2005. Outliers Statistical Treatment of Analytical Data. CRC Press, pp. 68–70.

Bigot, A., Minguez, L., Giambérini, L., Rodius, F., 2011. Early defense responses in the freshwater bivalve *Corbicula fluminea* exposed to copper and cadmium: transcriptional and histochemical studies. Environmental Toxicology 26, 623–632.

Blaser, S.A., Scheringer, M., MacLeod, M., Hungerbühler, K., 2008. Estimation of cumulative aquatic exposure and risk due to silver: contribution of nano-functionalized plastics and textiles. Science of the Total Environment 390, 396–409.

Bouwmeester, H., Poortman, J., Peters, R.J., Wijma, E., Kramer, E., Makama, S., Puspitaninganindita, K., Marvin, H.J.P., Peijnenburg, A.A.C.M., Hendriksen, P.J.M., 2011. Characterization of translocation of silver nanoparticles and effects on whole-genome gene expression using an in vitro intestinal epithelium coculture model. ACS Nano 5, 4091–4103.

Brulle, F., Mitta, G., Cocquerelle, C., Vieau, D., Lemiere, S., Lepretre, A., Vandenbulcke, F., 2006. Cloning and real-time PCR testing of 14 potential biomarkers in *Eisenia fetida* following cadmium exposure. Environmental Science and Technology 40, 2844–2850.

Brulle, F., Mitta, G., Leroux, R., Lemiere, S., Lepretre, A., Vandenbulcke, F., 2007. The strong induction of metallothionein gene following cadmium exposure transiently affects the expression of many genes in *Eisenia fetida*: a trade-off mechanism? Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 144, 334–341.

Burgos, M.G., Winters, C., Sturzenbaum, S.R., Randerson, P.F., Kille, P., Morgan, A.J., 2005. Cu and Cd effects on the earthworm *Lumbricus rubellus* in the laboratory: multivariate statistical analysis of relationships between exposure, biomarkers, and ecologically relevant parameters. Environmental Science and Technology 39, 1757–1763.

Butterfield, D.A., 1997. Beta-amyloid-associated free radical oxidative stress and neurotoxicity: implications for Alzheimer's disease. Chemical Research in Toxicology 10, 495–506.

Chae, Y.J., Pham, C.H., Lee, J., Bae, E., Yi, J., Gu, M.B., 2009. Evaluation of the toxic impact of silver nanoparticles on Japanese medaka (*Oryzias latipes*). Aquatic Toxicology 94, 320–327.

Choi, J.E., Kim, S., Ahn, J.H., Youn, P., Kang, J.S., Park, K., Yi, J., Ryu, D.-Y., 2010. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquatic Toxicology 100, 151–159.

Di Giulio, R.T., Benson, W.H., Sanders, B.M., Van Veld, P.A., 1995. Biochemical mechanisms: metabolism, adaptation, and toxicity. In: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology: Effects, Environmental Fate and Risk Assessment. Taylor & Francis, Bristol, USA, pp. 523–561.

Drake, J., Link, C.D., Butterfield, D.A., 2003. Oxidative stress precedes fibrillar deposition of Alzheimer's disease amyloid beta-peptide (1-42) in a transgenic *Caenorhabditis elegans* model. Neurobiology of Aging 24, 415–420.

Flick, K., Kaiser, P., 2012. Protein degradation and the stress response. Seminars in Cell & Developmental Biology 23, 515–522.

Gopalakrishna, R., Jaken, S., 2000. Protein kinase C signaling and oxidative stress. Free Radical Biology and Medicine 28, 1349–1361.

Gottschalk, F., Sonderer, T., Scholz, R.W., Nowack, B., 2009. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. Environmental Science & Technology 43, 9216–9222.

Hardas, S.S., Butterfield, D.A., Sultana, R., Tseng, M.T., Dan, M., Florence, R.L., Unrine, J.M., Graham, U.M., Wu, P., Grulke, E.A., Yokel, R.A., 2010. Brain distribution and toxicological evaluation of a systemically delivered engineered nanoscale ceria. Toxicological Sciences 116, 562–576.

Ho, C.M., Yau, S.K.W., Lok, C.N., So, M.H., Che, C.M., 2010. Oxidative dissolution of silver nanoparticles by biologically relevant oxidants: a kinetic and mechanistic study. Chemistry – An Asian Journal 5, 285–293.

Homa, J., Olchawa, E., Stürzenbaum, S.R., John Morgan, A., Plytycz, B., 2005. Early-phase immunodetection of metallothionein and heat shock proteins in extruded earthworm coelomocytes after dermal exposure to metal ions. Environmental Pollution 135, 275–280.

Judy, J.D., Unrine, J.M., Bertsch, P.M., 2010. Evidence for biomagnification of gold nanoparticles within a terrestrial food chain. Environmental Science & Technology 45, 776–781.

Kiliç, G.A., 2011. Histopathological and biochemical alterations of the earthworm (*Lumbricus terrestris*) as biomarker of soil pollution along Porsuk River Basin (Turkey). Chemosphere 83, 1175–1180.

Kim, S., Choi, J.E., Choi, J., Chung, K.-H., Park, K., Yi, J., Ryu, D.-Y., 2009. Oxidative stress-dependent toxicity of silver nanoparticles in human hepatoma cells. Toxicology in Vitro 23, 1076–1084.

Lim, D., Roh, J.-y., Eom, H.-j., Choi, J.-Y., Hyun, J., Choi, J., 2012. Oxidative stress-related PMK-1 P38 MAPK activation as a mechanism for toxicity of silver nanoparticles to reproduction in the nematode *Caenorhabditis elegans*. Environmental Toxicology and Chemistry 31, 585–592.

Luoma, S.N., 2008. Silver Nanotechnologies and the Environment: Old Problems or New Challenges? Woodrow Wilson International Center for Scholars, Washington.

Mueller, N.C., Nowack, B., 2008. Exposure modeling of engineered nanoparticles in the environment. Environmental Science & Technology 42, 4447–4453.

Nel, A.E., Mädler, L., Velegol, D., Xia, T., Hoek, E., Somarsundaran, P., Klaessig, F., Castranova, V., Thompson, M., 2009. Understanding biophysicochemical interactions at the nano–bio interface. Nature Materials 8, 543–557.

Ouyang, H., Vogel, H.J., 1998. Metal ion binding to calmodulin: NMR and fluorescence studies. BioMetals 11, 213–222.

Park, K., Park, E.-J., Chun, I., Choi, K., Lee, S., Yoon, J., Lee, B., 2011. Bioavailability and toxicokinetics of citrate-coated silver nanoparticles in rats. Archives of Pharmacal Research 34, 153–158.

Parsell, D.A., Lindquist, S., 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annual Review of Genetics 27, 437–496.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 30.

Roh, J.-y., Sim, S.J., Yi, J., Park, K., Chung, K.H., Ryu, D.-y., Choi, J., 2009. Ecotoxicity of silver nanoparticles on the soil nematode *Caenorhabditis elegans* using functional ecotoxicogenomics. Environmental Science & Technology 43, 3933–3940.

Shoults-Wilson, W., Reinsch, B., Tsyusko, O., Bertsch, P., Lowry, G., Unrine, J., 2011a. Role of particle size and soil type in toxicity of silver nanoparticles to earthworms. Soil Science Society of America Journal 75, 365–377.

Shoults-Wilson, W.A., Reinsch, B.C., Tsyusko, O.V., Bertsch, P.M., Lowry, G.V., Unrine, J.M., 2011b. Effect of silver nanoparticle surface coating on bioaccumulation and reproductive toxicity in earthworms (*Eisenia fetida*). Nanotoxicology 5, 432–444.

Shoults-Wilson, W.A., Zhurbich, O.I., McNear, D.H., Tsyusko, O.V., Bertsch, P.M., Unrine, J.M., 2011c. Evidence for avoidance of Ag nanoparticles by earthworms (*Eisenia fetida*). Ecotoxicology 20, 385–396.

Spurgeon, D.J., Stürzenbaum, S.R., Svendsen, C., Hankard, P.K., Morgan, A.J., Weeks, J.M., Kille, P., 2004. Toxicological, cellular and gene expression responses in earthworms exposed to copper and cadmium. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 138, 11–21.

Stürzenbaum, S.R., Kille, P., Morgan, A.J., 1998. The identification, cloning and characterization of earthworm metallothionein. FEBS Letters 431, 437–442.

Sultana, R., Ravagna, A., Mohmmad-Abdul, H., Calabrese, V., Butterfield, D.A., 2005. Ferulic acid ethyl ester protects neurons against amyloid beta-peptide(1-42)-induced oxidative stress and neurotoxicity: relationship to antioxidant activity. Journal of Neurochemistry 92, 749–758.

Tsyusko, O., Unrine, J.M., Spurgeon, D.J., Blalock, E.M., Starnes, D., Tseng, M.T., Joice, G., Bertsch, P.M., 2012. Toxicogenomic responses of the model organism *Caenorhabditis elegans* to gold nanoparticles. Environmental Science & Technology 46, 4115–4124.

Unrine, J.M., Hunyadi, S.E., Tsyusko, O.V., Rao, W., Shoults-Wilson, W.A., Bertsch, P.M., 2010a. Evidence for bioavailability of Au nanoparticles from soil and biodistribution within earthworms (*Eisenia fetida*). Environmental Science & Technology 44, 8308–8313.

Unrine, J.M., Tsyusko, O.V., Hunyadi, S.E., Judy, J.D., Bertsch, P.M., 2010b. Effects of particle size on chemical speciation and bioavailability of copper to earthworms (*Eisenia fetida*) exposed to copper nanoparticles. Journal of Environmental Quality 39, 1942–1953.

USEPA, 2009. Targetted National Sewage Sludge Report: Overview Report, Washington, DC.

Valko, M., Morris, H., Cronin, M.T.D., 2005. Metals, toxicity and oxidative stress. Current Medicinal Chemistry 12, 1161–1208.

Yang, X., Gondikas, A.P., Marinakos, S.M., Auffan, M., Liu, J., Hsu-Kim, H., Meyer, J.N., 2011. Mechanism of silver nanoparticle toxicity is dependent on dissolved silver and surface coating in *Caenorhabditis elegans*. Environmental Science & Technology 46, 1119–1127.

Yokel, R.A., Florence, R.L., Unrine, J.M., Tseng, M.T., Graham, U.M., Wu, P., Grulke, E.A., Sultana, R., Hardas, S.S., Butterfield, D.A., 2009. Biodistribution and oxidative stress effects of a systemically-introduced commercial ceria engineered nanomaterial. Nanotoxicology 3, 234–248.

Zhang, Y., Shen, G., Yueshu, Y., Hongling, Z., 2009. The hormetic effect of cadmium on the activity of antioxidant enzymes in the earthworm *Eisenia fetida*. Environmental Pollution 157, 3064–3068.